ANTIOXIDANT COUNTERMEASURE TO MUSCLE DISUSE ATROPHY

By

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Dedicated to my father

Robert J. Koesterer

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TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iii

CHAPTER 1 INTRODUCTION AND REVIEW OF LITERATURE 1

- Introduction 1
- Review of Literature 3
  - Hindlimb Unweighting Model 3
  - Loss of Muscle Protein 3
    - Protein synthesis 5
    - Protein degradation 6
  - The Role of Proteases in Muscle Atrophy 7
  - The Role of Reactive Oxygen Species in Muscle Atrophy 7
  - Antioxidant Defense Systems 9
    - Vitamin E 10
    - Vitamin C 12
    - Glutathione 13
    - Selenium 13
    - Carotenoids 14
    - Flavonoids 15
    - Ubiquinones 16
- Summary 17
- Purpose 18
- Rationale 18
- Questions 19
- Hypotheses 19

CHAPTER 2 METHODS 20

- Animals 20
- Pre-experimental Period 20
- Diet 21
- Experimental Period 21
- Data Collection 22
  - *In situ* Protocol 22
  - Measurement of contractile properties 23
    - Force and one-half relaxation time (1/2 RT) 23
    - Force / velocity protocol 24

- Tissues Removal 24
- Biochemical Analysis 24
  - Wet/dry weight 24
  - Isolation of myofibrillar protein 25
  - Thiobarbituris acid-reactive substance (TBARS) 25
  - Protein carbonyls 25

In Vitro Measurement of Tissue Antioxidant Capacity 26
- Hydroxyl Radical System 26
- Hypoxanthine-Xanthine Oxidase System 27
- Peroxyl Radical System 27

Statistical Analysis 27

CHAPTER 3 RESULTS 28

Overview of Experimental Findings 28
Antioxidant Supplementation 28
Protein Carbonyls 29
Lipid Peroxidation 30
Wet Muscle Weights 30
Gastrocnemius Contractile Function 30
Muscle Protein Concentration and Water Content 31
In Vitro Oxidative Challenges 31

CHAPTER 4 DISCUSSION 41

Overview of Experimental Findings 41
Antioxidant Supplementation and Skeletal Antioxidant Capacity 41
Protein Oxidation and Lipid Peroxidation 42
Muscle Weight and Body Weight 43
Contractile Properties 44
Protein Concentration and Water Content 44
Why No effect from Antioxidant Supplementation 45
Summary and Conclusions 47

LIST OF REFERENCES 48

BIOGRAPHICAL SKETCH 59

Abstract of Dissertation Presented to the Graduate School
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ANTIOXIDANT COUNTERMEASURE TO MUSCLE DISUSE ATROPHY

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Muscular atrophy is a significant problem during periods of prolonged space flight, extended bedrest, and orthopedic injuries. In order to develop countermeasures to attenuate muscle atrophy, it is necessary to understand the mechanisms of how muscle atrophy occurs. Much of muscle wasting is due to protein loss, the majority of which is from protein degradation. Oxidation of proteins "marks" them for degradation by most proteases. A couple of studies have demonstrated an increase in oxidative stress during muscle atrophy resulting from immobilization. These studies have used vitamin E to decrease the levels of oxidative stress and attenuate muscle atrophy in the immobilization model. To date no experiments have evaluated the ability of antioxidant supplementation to attenuate oxidative stress and muscle atrophy resulting from hindlimb unweighting (HLU). The purpose of this study was to determine if oxidative stress is increased during HLU, and if an antioxidantsupplementation will attenuate the muscular atrophy and changes in contractile properties resulting from HLU. Protein carbonyls, an indicator of oxidataive stress, increased 44% with HLU. A 44% decrease in muscle mass, a 7% decrease in body weight and 28% decrease in tetanic force with HLU were consistent with exposure to unweighting. Antoxidants did not attenuate the muscle atrophy during HLU since the supplemented group showed a 46% decrease in muscle mass. The muscles were subjected to three different oxidative challenges, and an increased antioxidant protection was demonstrated in the antioxidant supplemented groups. These data indicate that the antioxidant supplementation was not an effective countermeasure to the atrophy associated with HLU.

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Muscle atrophy due to unweighting is a significant problem during periods of prolonged space flight, extended bedrest, and orthopedic injuries. In order to develop countermeasures to attenuate this problem, it is necessary to understand the mechanisms of how muscle atrophy occurs. The loss of muscle protein could be due to a decrease in protein synthesis, an increase in protein degradation or both. To date, most studies addressing the problem of muscle atrophy have examined the mechanisms of decreased protein synthesis. However, it is now apparent that increased protein degradation is a major factor in causing the atrophy. Only a limited number of studies have addressed the issue of what mechanisms may be responsible for the degradation.

Protein synthesis rate in the unweighted soleus decreases within 5 hours of unweighting, and may decrease 50-60% over 7-14 days (Booth & Criswell, 1997). The mechanisms responsible for this decreased protein synthesis are due to a slowing of protein translation (Ku & Thompson, 1994).

Protein degradation begins at a much slower rate, peaks after ~14 days, and then returns to normal levels after ~24 days (Thomason et al. , 1989). The peak rate of degradation (% degraded/day) is approximately 200% higher than normal (Thomason et al., 1989). Oxidative modification of protein "marks" these proteins for degradation by most common proteases (Stadtman, 1993; Rivett, 1985). An increase in protein carbonyls is a measure of proteins which have been oxidatively modified (Yan et al. , 1996; Oliver et al., 1987; Amici et al., 1989). This increase has been demonstrated with processes of increased protein degradation (Berlett & Stadtman, 1997; Stadtman, 1990 & 1993; Levine et al., 1990; Rivett, 1985). Therefore, an increased oxidative stress could result in increase protein oxidation and subsequent degradation by proteolytic enzymes.

Kondo has demonstrated an increase in oxidative stress during muscle atrophy resulting from the immobilization (Kondo et al., 1991). Further, Kondo performed a series of studies in the early 90's that developed into a scheme for the increase in oxidative stress with immobilization. Pilot data from our lab have indicated an increase in oxidative stress in hindlimb unweighting (HLU) skeletal muscle. The lipid soluble antioxidantvitamin E has been used to decrease the levels of oxidative stress and attenuate muscle atrophy in the immobilization model (Kondo & Itokawa, 1994; Appell et al., 1997). To date no experiments have evaluated the ability of an antioxidantsupplemented diet to attenuate oxidative stress and muscle atrophy resulting from HLU.

Atrophy associated with HLU also has significant functional consequences as demonstrated through alterations in contractile properties of skeletal muscle. There is a decrease in the tetanic force generating capacity (Thomason & Booth, 1990). HLU also causes a shift towards faster muscle fiber types, which can be demonstrated by an increase in maximum velocity of contraction (V_{MAX}) and decrease in half-relaxation time (1/2 RT) (Thomason & Booth, 1990) .

Review of Literature

Hindlimb Unweighting Model

Hindlimb unweighting (HLU) of the rat is a well accepted model for muscle unloading which simulates the microgravity of space flight (Ohira et al., 1992), and has been shown to cause changes in muscle size, biochemistry, and function similar to those associated with microgravity (McDonald et al., 1994). Decreasing the load on a muscle by removing weight-bearing causes atrophy (Thomason & Booth, 1990). The mechanisms that cause atrophy are not clear, but are apparently linked to the unweighting as well as to hormonal alterations (Thomason & Booth, 1990; Booth & Criswell, 1997).

HLU also alters *in situ* contractile properties and myosin heavy chain (MHC) composition of the rat. Peak tetanic force and one-half relaxation time (1/2 RT) are reduced, while maximum shortening velocity (V_{MAX}) increases after HLU (Fitts et al., 1986; McDonald et al., 1994). MHC isoforms have been shown to shift from slow to fast (Diffe et al., 1991; McDonald et al., 1994).

Loss of Muscle Protein

During hindlimb unweighting, there is a preferential atrophy of slow-twitch muscle fibers (Desplanches et al., 1987; Haida et al., 1989; Reiser et al, 1987; Steffen & Musacchia, 1984). This loss of muscle mass is due to a decrease in fiber diameter with no loss in fiber number (Templeton et al., 1988; Thomason et al., 1987). Several studies have shown that most of the atrophy (~90%) occurs during the first two weeks of HLU (Desplanches et al., 1987; Haida et al., 1989; Reiser et al., 1987). This loss of muscle is due mostly to protein loss, most of which is due to protein degradation (Thomason & Booth, 1990) (Figure 1).

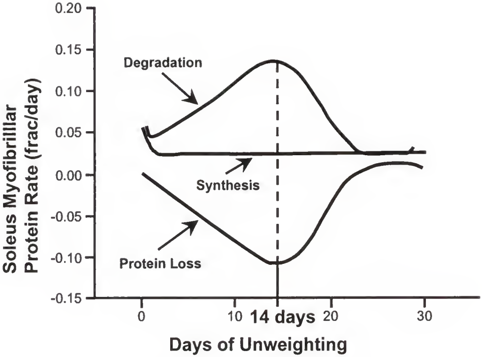

Figure 1. Muscle alterations with unweighting (Thomason & Booth, 1990)

The loss of total protein in the slow twitch soleus muscle has been shown to range from 26-58% over a period of 3-14 days (Steffen & Musacchia, 1984; Loughna et al., 1986; Goldspink et al., 1986; Babij & Booth, 1988). In contrast, the mixed fiber gastrocnemius lost only 13-31% over that same time period (Desplanches et al., 1987; Steffen & Musacchia, 1984; Geoghegan et al., 1989). Because predominantly slow twitch skeletal muscle seems to be most affected by HLU, the soleus muscle is most often used to determine the effects of HLU on muscle.

The concentration of protein in the soleus, as opposed to the total amount, is perhaps a more important measure of functional status of the muscle during atrophy since the amount of protein / unit muscle weight is a better predictor of muscle force generating ability (Kandarian & White, 1989). Protein concentration in the soleus has been shown to decrease up to 30% over a period of 2-6 weeks (Flynn & Max, 1985; Tsika et al., 1987). This indicates that most of the decrease in muscle mass is due to a decrease in muscle protein. The initiation of this loss of protein has been shown to begin within five hours of HLU (Thomason et al., 1989).

Even more important to contractile function of the muscle may be the amount of myofibrillar protein lost in relation to the total protein. Indeed, there appears to be a preferential loss of myofibrillar protein in relation to total protein (Thomason et al., 1987; Jaspers et al., 1985 & 1986). Thomason et al. (1987) have shown that adult rats lost 80% of myofibrillar protein in the soleus after 4 weeks of HLU, whereas the plantaris muscle lost only 29% of myofibrillar protein after 6 weeks (Tsika et al, 1987). In addition, these authors (Thomason et al., 1987) have shown that the concentration of myofibrillar protein (per gram of wet muscle weight) in soleus muscle decreases ~50% in four weeks in contrast to the plantaris muscle which decreases mass, sarcoplasmic and myofibrillar protein in equal proportions (Tsika et al., 1987). Further, in comparing the sarcoplasmic protein loss to the myofibrillar protein loss, Jaspers et al. (Jaspers et al., 1988) found sarcoplasmic protein did not decrease whereas myofibrillar protein decreased ~20% in the same muscles.

Protein synthesis

There is some confusion concerning the rate of total protein synthesis during HLU because several studies have used young, growing animals as a model. These studies have shown protein synthesis to be decreased about 20% (Loughna et al., 1986; Goldspink et al., 1986; Loughna et al., 1987) whereas synthesis rates in mature, adult animals are depressed 45% (Thomason et al., 1989). In addition, myofibrillar protein synthesis rates are decreased 59% (Thomason et al., 1989). It seems that the decrease in protein synthesis during HLU is caused by a decrease in protein translation and not by a decrease in protein transcription (Ku & Thomason, 1994). While it is obvious from the findings of these studies that protein synthesis is decreased during HLU the magnitude of decrease and the mechanism, which initiates the decreased protein synthesis, remain obscure.

Protein degradation

As with protein synthesis, the rate of protein degradation has not been accurately assessed during HLU. One reason is that true rates of protein degradation are much more difficult to determine than rates of protein synthesis. There are several methods in use to estimate the rate of muscle protein degradation. First, by measuring the amino acid release into the bathing medium of *in vitro* mounted excised muscle, several studies have shown a 45-55% increase in amino acid release in HLU soleus muscle (Jaspers et al., 1985 & 1989; Goldberg, 1980; Tischler, 1981; Furuno et al., 1990). A second approach has been to calculate the fractional rates of protein degradation by subtracting the mean growth rate from the mean fractional rate of protein synthesis *in vivo* (Geoghegan et al., 1989; Loughna et al., 1987). These studies have estimated protein degradation rates to be increased 55-240% in the soleus over a one week period (Loughna et al., 1986; Goldspink et al., 1986; Jaspers et al., 1988). In addition, in the study which found the lowest rate of protein degradation (Jaspers et al., 1988), it was also shown that the extensor digitorum longus (EDL) muscle (a fast, phasically activated muscle) did not atrophy and the rate of protein degradation was zero. These data add further evidence to the notion that the slow soleus muscle and the fast EDL muscle are fundamentally different in their response to HLU. A third approach has been to make serial measures of myofibrillar protein content over a period of days and, through computer modeling, determine the rate of protein degradation throughout the hindlimb unweighting period (Thomason et al., 1989). The drawback to this method is that it gives derived estimates rather than measured. However, the results produced using this method show protein degradation rates to be ~50%; similar to those found *in vitro* using young, growing animals (Jaspers & Tischler, 1986).

Thus, even if the lowest rates of protein degradation reported are considered, it is likely that protein degradation is substantial during HLU. Indeed, most studies have concluded that increased protein degradation contributes much more to muscle atrophy than does a decrease in protein synthesis. Figure 1 illustrates the likely contribution of myofibrillar protein synthesis and degradation to the overall levels of myofibrillar protein in soleus muscle during HLU (Thomason et al., 1989).

The Role of Proteases in Muscle Atrophy

Although is clear that muscle proteases play an integral role in the breakdown of muscle proteins, it is not clear as to their exact function or the order in which they may act (Goll et al., 1989). The classes of proteases that are most important for skeletal muscle breakdown are lysomal, calpains, and ubiquitin. Most evidence suggests that all muscle proteolytic systems are activated during atrophy from HLU, but it does seem that the ATP-ubiquitin-dependent pathway is the primary system responsible for protein degradation (Mitch & Goldberg, 1996). Recent information suggests that the role of these proteases in the breakdown of muscle during HLU plays a significant role in several steps leading to the release of free amino acids.

The Role of Reactive Oxygen Species in Muscle Atrophy

It has recently been shown that immobilization of muscle results in oxidative damage (Kondo & Itokawa, 1994). Kondo & Itokawa (1994) demonstrated that thiobarbituric acid-reactive substance (TBARS), a marker of lipid peroxidation, increases 60% during 12 days of hindlimb immobilization. This is significant because strong evidence exists that exposure of muscle to oxidizing species results in a reduction of muscle mass and force production (Kondo & Itokawa, 1994; Kolbeck et al., 1997; Shindoh et al., 1990; Reid et al., 1992; Brotto & Nosek, 1996). Oxidative modification of proteins “marks’ them for degradation by proteolysis (Stadtman, 1993; Rivett, 1985). Proteolytic enzymes will increase the rate of protein degradation and result in a decrease in the protein concentration. The mechanism responsible for this oxidant-induced damage is unclear but is probably linked to damage of oxidant-sensitive structural and contractile proteins (Castilho et al., 1996; Reid, 1996; Lawler & Powers, 1998). There are several mechanisms that could explain how oxidative stress accelerates muscle atrophy.

First, the plasma membrane and sarcoplasmic reticulum are damaged by lipid peroxidation, allowing calcium to enter the cell (Mourelle & Meza, 1991). Indeed, Ca^{2+}-ATPase is inhibited by lipid peroxidation. In addition, Ca^{2+} has been shown to stimulate phospholipid hydrolysis and non-lysosomal proteolysis (Nicotera et al., 1986; Pascoe & Reed, 1989).

Second, free radical damage to lysosomal membrane causes the leakage of lysosomal proteases into the cytoplasm (Mak et al., 1983). In fact, there is evidence that lysosomal proteases increase in muscular atrophy (Max et al., 1971).

Finally, radical damage to proteins makes them more susceptible to proteolysis (Stadtman, 1993). Kondo has demonstrated an increase in Cu-Zn SOD, which dismutates superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) , and would indicate an increased level of $O_2^{\bullet-}$ in the cytoplasm. He speculated that this increase in $O_2^{\bullet-}$ was due to an increased activity of xanthine oxidase (XOD) resulting from an increased nucleotide degradation. The increase in intracellular calcium also would activate proteases which would convert XOD to the $O_2^{\bullet-}$ producing isoform. Glutathione peroxidase (GPX) and Catalase (CAT), which converts H_2O_2 to H_20, did not increase in the cell. Therefore the cell is producing $O_2^{\bullet-}$ and converting it to H_2O_2, resulting in increased levels of both in the cell. Kondo further demonstrated an increase in Fe^{2+} in the cell, which can lead to production of the highly reactive hydroxyl radical ($HO^{\bullet}$) through either Fenton chemistry or the Haber-Weiss reaction. These radicals can all lead to oxidation of lipid, protein, and DNA.

It is unknown which of these or other mechanisms may begin the cascade of oxidative events, but there is strong evidence that the process can be accelerated rapidly. Although evidence is mounting that free radical damage occurs during immobilization of muscle, it is unknown if these mechanisms are activated in unweighted muscle.

Antioxidant Defense Systems

Given the potential role of reactive oxygen species in muscle atrophy, it is not surprising that muscle cells have extensive protective systems against damage from radicals. These protective components are classified as the "antioxidant defense systems" (Yu, 1994). Primary defense system includes antioxidantscavenging enzymes and non-enzymatic compounds (Yu, 1994; Davies et al., 1971). Antioxidantenzymes include (1) superoxide dismutase (SOD), which promotes the dismutation of superoxide ($O_2^{\bullet-}$) to H_2O_2 and oxygen; (2) glutathione peroxidase (GPX), which reduces H_2O_2 to oxidized glutathione (GSSG) and water with the use of glutathione (GSH); (3) catalase (CAT), which converts H_2O_2 to water and oxygen. Non-enzymatic compounds include vitamin E, vitamin C, carotenoids, glutathione, ubiquinones, and flavonoids (Powers & Hamilton, 1999; Halliwell & Gutteridge, 1999; Yu, 1994). Secondary defense consists of lipolytic enzymes, phospholipases, proteolytic enzymes, proteases, peptidase, DNA repair enzymes, endonuclease, exonuclease, and ligase (Yu, 1994; Davies et al, 1971). Anti-oxidants work not only individually, but also with an additive and synergistic interactions in order to maintain redox homeostasis (Chen & Tappel, 1994; Powers & Hamilton, 1999; Yu, 1994).

Countermeasures designed to increase the cells' antioxidant defenses are a promising strategy for HLU associated muscle atrophy . Although increase in enzymatic anti-oxidants can be achieved following stresses including exercise training (Powers et al., 1993 & 1999), an effective method of increasing antioxidantdefenses is through supplementation with non-enzymatic anti-oxidants, which results in the elevation of cellular antioxidant concentration, resulting in increased protection against oxidative damage (Chen & Tappel, 1994). The nonenzymatic antioxidants employed in the current study and their roles in defending against reactive oxygen species (ROS) damage are reviewed in the next sections.

Vitamin E

Vitamin E is the most widely distributed antioxidant, and is the primary chain-breaking molecule in lipophilic environment of cell membranes (Burton & Traber, 1990). The term vitamin E refers to at least eight different structural isomers of tocopherol (Yu, 1994; Bjorneboe et al., 1990). α-Tocopherol is believed to possess the most potent antioxidant activity of these (Burton & Traber, 1990). The antioxidant action of α-tocopherol is highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxyl radicals (Ross & Moldeus, 1991). Protection of membranes by vitamin E is dependent upon the incorporation of α-tocopherol into cellular membranes (Tappel, 1972).

While vitamin E is an efficient radical scavenger, the interaction of vitamin E with a radical results in a reduction of functional vitamin E and the formation of a vitamin E radical. However, the vitamin E radical can be recycled back to its native state by a variety of other antioxidants such as vitamin C and glutathione (Burton & Traber, 1990; Kagan et al., 1992; Scholich et al., 1989). It is postulated that the ability of vitamin E to serve as an antioxidant is synergistically connected to these other antioxidants (Bendich et al., 1986).

Under most dietary conditions the concentration of vitamin E in tissues is relatively low (Janero, 1991). For example, vitamin levels in biological membrane is usually less than 0.1 nmol per mg of membrane protein or, in other words, one molecule per 1000-2000 membrane phospholipid molecules (Packer et al., 1995). Since membrane levels of vitamin E are low, numerous investigators have attempted to elevate tissue levels by either infusion or dietary supplementation. At high doses however, the intestinal absorption of dietary vitamin E decreases (Losowsky et al., 1972). As the oral dose of radioactive alpha tocopherol increases, the percent absorption decreases from 70% at RDA levels (75 IU/kg diet) to 30% at a very high dose of 20,000 IU/kg diet (Davies et al., 1971). Vitamin E has a relatively long biological half-life (2.4 days) in the rat (Machlin & Gabriel, 1982) and has few, if any, side effects even when administered in megadoses (Burton & Traber, 1990; Machlin & Gabriel, 1982). Machlin and Gabriel

(1982) fed rats 10,000 IU vitamin E/kg diet for up to 20 weeks and significantly elevated tissue and serum tocopherol concentrations with no signs of toxicity. Kondo (1991) and Appell (1997) injected vitamin E intraperitoneally and demonstrated a decreased level of oxidative stress in skeletal muscle.

Vitamin C

Vitamin C (ascorbic acid or ascorbate) is a hydrophilic antioxidant that exerts its radical scavenging activities in the aqueous compartments of cells (Yu, 1994). Vitamin C scavenges superoxide, peroxyl, and hydroxyl radicals directly, and also plays an important role in recycling the vitamin E radical back to its active form (Chen, 1989; Packer et al., 1979; Yu, 1994). Like vitamin E, in the process of scavenging radicals the vitamin C radical is generated and must be recycled to its reduced state to continue exerting its antioxidant activities. Vitamin C can act as both an antioxidant and a pro-oxidant (Bendich et al., 1984 & 1986; Buettner, 1986). Excess amounts of (> 1 mM) may act a pro-oxidant in the presence of the transition metal Fe3+, resulting in the reduction to Fe^{2+}, which can induce radical generation (Yu, 1994). Therefore, careful consideration must be given to vitamin C dosage when supplementing for the purpose of preventing oxidative damage.

The protective effects of supplemental vitamin C, as well as synergistic effects of vitamins C and E, have been investigated extensively. Supplementation with vitamin C has been shown to increase the global antioxidant capacity and protect a variety of tissues and cells against oxidative injury (Maellaro et al., 1994). Supplementation with both vitamins C and E appears to lead to a sparing effect on vitamin E as well as protection against oxidative injury (Chen, 1989; Packer et al., 1979; Tanaka et al., 1995). Accordingly, vitamin C deficiency induces a greater susceptibility to oxidative injury, perhaps to a greater extent than does vitamin E deficiency (Tanaka et al., 1995). Further, supplementation with a combination of vitamins C and E may help prevent the potential pro-oxidant effects of larger doses of vitamin C (Chen, 1989).

Glutathione

Glutathione (GSH), a tripeptide, γ-glutamyl-cysteine-glycine, is the most abundant nonprotein thiol in cells (Meister and Anderson, 1983). GSH serves multiple roles in defending against oxidative damage. Perhaps its most important role is as a substrate for glutathione peroxidase (GPX) which catalyses the enzymatic reduction of hydrogen peroxide and other organic peroxides to water and alcohol, respectively. In this enzymatic process, two molecules of GSH are oxidized to form the disulfide form of glutathione (GSSG). GSSG is then recycled back to the reduced form by glutathione reductase with NADPH as a substrate. (Ji, 1995; Ji et al.1992). GSH can also directly scavenge hydroxyl, superoxide, and carbon based radicals, as well as singlet oxygen (Yu, 1994). Finally, GSH has an important role in recycling the radical forms of other antioxidants (i.e., vitamins E and C) to restore their radical-quenching capacity (Packer et al., 1991).

Given the many functions of GSH in maintaining a reduced cellular environment, it is not surprising that alterations in GSH content can alter cell function. GSH and N-acetyl cysteine (NAC; a GSH-replenishing drug) administered intraperitoneally, have been shown to result in increases in tissue GSH concentration and improvements in muscle endurance (Reid et al., 1994; Shindoh et al., 1990).

Selenium

Selenium is a trace nutrient and serves as an active site for glutathione peroxidase (GPX) (Tappel, 1980; Forstrom et al., 1978). Although selenium's protective action has not been defined, it is thought to be related to the selenium requirement for the optimal activity of selenium-dependent GPX. The activity of GPX correlates well with the concentration of selenium in blood. A deficiency in selenium alters the antioxidant defense systems by depressing GPX (Yu, 1994). Ji et al. (1988) found that selenium deficiency depleted GPX in both liver and skeletal muscle.

Carotenoids

Carotenoids have long been considered antioxidants because of their ability to scavenge free radicals (Krinsky, 1979a & 1982; Krinsky & Deneke, 1982). Carotenoids quench free radicals and other ROS (e.g., singlet oxygen), and protect against lipid peroxidation (Foote, 1979; Foote & Denny, 1988; Krinsky, 1979a). β-carotene exhibits antioxidant activity through its inhibition of lipid peroxidation induced by xanthine oxidase (Krinsky & Deneke, 1982). Many carotenoids are also chain-terminating antioxidants. The antioxidant activity of β-carotene, lycopene, astaxanthin, and canthaxanthin has been well-documented (Rousseau et al., 1992). There is some controversy as to which carotenoid has the greatest radical scavenging activity.

β-carotene has long been thought to be the most effective. However, Rousseau et al. (1992) states that carotenoids with oxo groups at the 4 and 4`-positions in the beta-ionine ring like canthaxanthin and astaxanthin are more effective scavengers than β-carotene.

In contrast, other investigations have found β-carotene and lycopene to have greater scavenging activity than astaxanthin, and canthaxanthin (Miller et al., 1996). *In vivo* assessment techniques are limited by the lack of good models, and *in vitro* by differing radical generating systems and methods for measuring scavenging activity may account for some of this controversy.

Carotenoids may act synergistically with β-carotene and α-tocopheral demonstrating distinct chain-breaking activities as oxygen tension varies. At low partial pressures of oxygen β-carotene is an efficient antioxidant, whereas α-tocopherol is more effective at higher partial pressures of oxygen (Burton, 1989; Rousseau et al., 1992). Whether β-carotene can actually recycle the vitamin E radical remains debated (Mayne & Parker, 1989; Rousseau et al., 1992). Similar to vitamin E, carotenoids with two polar groups distally situated on the molecule can span phospholipid membranes (i.e., β-carotene, astaxanthin, and canthaxanthin) and provide membrane stability (Rousseau et al., 1992).

β-carotene is relatively nontoxic even at plasma concentrations nearly 100-fold above normal levels. The only observed side effect in clinical trials has been hypercarotenodermia, a condition of yellowing of the skin (Bendich et al., 1988; Dinitrov et al., 1988). Canthaxanthin supplementation has been associated with reports of a reversible retinopathy (Harnois et al., 1989).

Flavonoids

Flavonoids are a family of diphenylpropanes (> 4000 identified) that commonly occur in plants which humans eat on a frequent basis. Family members include flavones, isoflavones, flavanones, anthocyanins, and catechin (Das, 1994). Some flavonoids have antilipoperoxidant, antitumoral, antiplatelet, anti-ischemic, anti-allergic, and anti-inflammatory activities. They may also inhibit an array of enzymes, including lipoxygenase, cycloosygenase, monooxygenase, xanthine oxidase, mitochondrial succinoxidase, and NADH-oxidase, phospholipase A2, and protein kinases. These effects are believed to be from the antioxidant properties of the flavanoids, including protection against iron induced free radical reaction, and inhibition of enzymes due to reactions with free radicals generated at the active site of these enzymes (Cao et al., 1997).

Radical scavenging activities of flavonoids appear to vary greatly among family members, but include quenching of peroxyl, hydroxyl, and superoxide radicals as well as hydrogen peroxide and a variety of chemically generated radicals not naturally found in the body (Cao et al., 1997; Cos et al., 1988).

Catechin, which is found in significant concentrations in green and black tea and red wine, is one of the flavonoids under investigation for their potential role in protecting against radical-mediated disease processes. Catechin has been shown to efficiently scavenge superoxide radicals, inhibit metal ion-mediated radical formation, and to inhibit formation of lipid peroxyl radical species (Salah et al., 1995; Sichel et al., 1991). Catechin has also been reported to prevent the radical-mediated depletion of vitamin E and β-carotene in human plasma in a dose-dependent manner (Lotito & Fraga, 1998).

Ubiquinones

Ubiquinone (coenzyme Q), in addition to its function as an electron and proton carrier in mitochondrial electron transport coupled to ATP synthesis, acts in its reduced form (ubiquinol) as an antioxidant. Ubiquinol is the only known lipid-soluble antioxidant that can be synthesized *de novo*, and for which there is an enzymatic mechanism to regenerate it from its oxidized state formed in the course of its antioxidant function. Ubiquinone-10 (coenzyme Q10) is the primary isoform in most mammalian species, and is found primarily in the reduced form, with approximately 50% of total cellular stores found in mitochondrial membrane (Ernster & Dallner, 1995; Aberg et al., 1992). Ubiquinol prevented both initiation and propagation of lipid peroxidation, protein oxidation (carbonyl formation) and DNA oxidation, and can also regenerate vitamin E radical (Ernster & Dallner, 1995).

Orally administered ubiquinone in humans increases the blood level of quinone (partly present as the quinol), and has the same effect in rats (Mohr et al., 1992; Zhang et al., 1995). Dietary supplementation with ubiquinone may act primarily by elevating the ubiquinone levels in blood. These elevated levels may enhance the protection of LDL from peroxidation, prevent radical damage caused by neutrophils in inflammatory diseases, and prevent oxidative injury by endothelial cells resulting from ischemia-reperfusion. These and other protective mechanisms function against radical damage taking place in the circulation may account for the majority of the reported benefits of ubiquinone (Ernster & Dallner, 1995). Coenzyme Q_{10} has the potential to reduce the overall oxidative level in the systemic circulation in our experiment.

Summary

In summary, immobilization has been demonstrated to increase oxidative stress and muscular atrophy. Whether the same mechanisms are active in HLU is yet to be determined. The oxidative stress may be increased due to calcium entering the cell, leakage of lysosomal proteases and radical production of $O_2^{\bullet -}$, H_2O_2 & $HO^{\bullet}$. Although it is unknown where the cascade may begin, there is strong evidence that once started the process of oxidative stress accelerates rapidly. Oxidative modification of proteins “marks” them for degradation by either lysomal, calpain, or ubiquitin proteolysis.

Antioxidant supplementation has the potential to reduce the level of oxidative stress in the muscle, reducing the amount of protein marked for degradation and ultimately attenuating the amount of atrophy from HLU.

Purpose

The purpose of this project was to determine if oxidative stress is increased during HLU, and if antioxidant supplementation will attenuate the muscular atrophy and functional changes in contractile properties resulting from unweighting.

Rationale

1) Kondo and Appell have previously demonstrated an increase in oxidative stress during immobilization, which can be attenuated by the administration of vitamin E (Kondo et al., 1991; Appell et al., 1997). Pilot data from our lab have indicated the same effects on oxidative stress during HLU which are also attenuated by an antioxidant supplementation. Therefore, it is speculated that lipid and protein oxidation will increase as a result of increased oxidative stress in the HLU groups.

2) Oxidative stress may be induced through calcium leakage, leakage of lysomal contents and radical production. This increased oxidative stress can modify proteins, marking them for degradation by lysomal, calpain, and ubiquitin proteolysis. It is speculated that protection from lipid peroxidation will be primarily due to increased muscle vitamin E, β-carotene, and coenzyme Q_{10} because they reside in the lipid phase of membranes. Supplementation with other compounds, including N-acetyl cysteine, vitamin C, and flavonoids, should provide protection against oxidative damage to cytosolic proteins such as cytoskeletal and contractile proteins. It would follow that the antioxidant supplementation will decrease oxidative stress, marking fewer proteins for

degradation, slowing the rate of protein degradation, resulting in an attenuation of muscular atrophy.

3) Although all muscle fibers atrophy during HLU, there is a preferential atrophy of slow-twitch muscle fibers, resulting in a decrease in force production, an increase in V_{MAX} and a decrease in ½ RT. A countermeasure should lessen the atrophy of the entire muscle, but there should be a greater capacity to diminish this effect in slow-twitch fibers.

Questions

1. Does oxidative stress accompany protein degradation resulting from HLU?

2. Will an antioxidant supplementation buffer the increase in oxidative radicals, and therefore attenuate the degradation and atrophy resulting from HLU?

3. Will an antioxidant supplementation attenuate the changes in contractile properties (e.g. decrease tetanic force, decrease ½ RT, and increase V_{MAX}) resulting from HLU?

Hypotheses

1. Lipid and protein oxidation measurements of oxidative stress will be increased in the HLU groups.

2. An antioxidant supplementation will attenuate the degree of seketal muscle atrophy resulting from HLU.

3. An antioxidant supplementation will attenuate the changes in skeletal muscle contractile properties (e.g. less decrease in tetanic force, less decrease in ½ RT, and less increase in V_{MAX}) resulting from HLU.

CHAPTER 2
METHODS

Animals

All experiments were performed using female Sprague-Dawley rats weighing between 245-255 grams upon arrival. The rat model was chosen for this study because 1) the nature of the study is invasive, 2) rat skeletal muscle is similar in composition and plasticity to human muscle, 3) previous research has examined the alterations in rat muscle with respect to this model, and 4) the cost effectiveness of using an animal model. This size of rats was chosen because protein synthesis and body mass has been shown to plateau at this weight (Pettersen et al. , 1996).

All animals were given water ad libitum, maintained on a 12 hour light/dark cycle, and handled daily in order to reduce contact stress with humans.

Pre-experimental Period

Upon arrival, all animals were provided a control diet for five days in order to stabilize body weight changes due to shipping. After body weights stabilized, the animals were randomly assigned by body weight to one of two groups (Control or Antioxidantsupplemented), and fed their respective diets for seven days. Concurrently, the antioxidantsupplemented animals were injected once a day with vitamin E (30 mg/kg IP). Vitamin E injections were given in the form of α-tocopherol solubilized in corn oil. The control diet animals were sham injected with a corresponding volume of vitamin E stripped corn oil (Purina).

Diet

Control diet animals were fed a diet designed to meet the National Research Council's recommended requirements (AIN93M) in the adult rat (Reeves et al., 1993). The antioxidantsupplemented animals were fed the AIN93M diet supplemented with the seven antioxidants listed in Table 1. The diets were professionally prepared (Bio-Serv, Frenchtown, NJ).

Table 1. Antioxidants supplemented to the AIN93M diet

Antioxidant	**per kg of rodent diet**
Vitamin E	1000 IU
Selenium	0.3 mg
Ascorbic acid	1000 mg
20% beta-carotene	225 mg
Coenzyme Q10	30 mg
N-acetyl cysteine	200 mg
± catechin	100 mg

Experimental Period

Control and Antioxidant animals were further randomly assigned by body weight to weight bearing or hindlimb unweighted groups to achieve the four following treatment groups;

Group 1) Weight bearing with Control Diet, C

Group 2) Weight bearing with Antioxidant supplementation, AO

Group 3) HLU with Control Diet, HLU-C

Group 4) HLU with Antioxidant supplementation, HLU-AO

All animals continued their respective diets during the 14 day experimental period. Vitamin E and sham injections were given every other day, at the same dosages as during pre-experimental period. The HLU and HLU-AO animals were hindlimb unweighted, while the C and AO animals remained weight bearing for the same 14 days. The tails of the HLU-C and HLU-AO rats were wrapped and connected to a swivel for suspension similar to the procedure described by Roer and Dillaman (1994) for 14 days. The rats were suspended from the top of a cage such that the hindlimbs were 1cm above the cage floor, and had complete (360^0) range of motion by use of the fore limbs. This afforded the animals free range of motion around the floor of the cage, but prevented climbing on the sides of the cage.

Data Collection

In situ Protocol

On day 14 of the experiment, the animals were anesthetized with sodium pentobarbital (30 mg/kg IP) with additional doses given as needed. After a surgical plane of anesthesia was reached, as determined by the absence of a reflex reaction, the animals were ventilated through an endotracheal tube with room air.

The soleus (SOL) of the right leg was dissected free, weighted and immediately frozen in liquid nitrogen for later biochemical analysis. The gastrocnemius (GS) muscle of the left hind limb was prepared *in situ* for contractile properties. The muscle was dissected free from overlying muscles and surrounding connective tissue, with care taken not to disrupt the blood supply. A bone pin was placed through the femur and the foot placed into a clamp to prevent movement. The GS tendon was attached to an isotonic force transducer (Cambridge model 400) with a lightweight chain. The Cambridge transducer is a combined force and position transducer. The force on the lever can be electronically controlled from 0 to 5000g while the length change is monitored or the length of the muscle can be held constant while the force is monitored. The transducer output was amplified and differentiated by operational amplifiers and underwent A/D conversion for analysis with a computer based data acquisition system (GW Instruments, Somerville, MA). The sciatic nerve was carefully isolated, tied, cut, and placed in a bipolar electrode connected to a square wave stimulator (Grass Instruments, model S48). The preparation was kept moist with saline covered with saline-soaked gauze and plastic wrap. The plastic wrap acted as a barrier to moisture loss and thus preventing the preparation from becoming dry during the experiment. The temperature was monitored throughout the experiment with a rectal probe and maintained near 37^0 C by means of a heating pad and lamp. Electrocardiograph (ECG) was monitored throughout the protocol.

Measurement of contractile properties

The following stimulation protocols were performed on the GS muscle. After 15 minutes of equilibration, the muscle was stimulated using a supramaximal square wave pulse (6V, 0.2-ms duration) delivered in 100-ms trains at 150 Hz. The muscle was repeatedly stretched and stimulated until the maximal isometric tetanic tension was obtained and the muscle was maintained at that length (L_O) throughout the experiment. At least two minutes was given between all stimulations.

Force and one-half relaxation time (1/2 RT)

Maximal isometric tetanic tension was determined by stimulating the muscle three separate times with the above parameters (6V - 0.2-ms duration - 100-ms trains - 150 Hz). Three independent twitch stimulations (6V - 0.2-ms duration - 150 Hz), were given to determine the maximal isometric twitch tension of the muscle. One-half relaxation time (½ RT) was determined by one stimulation at the twitch parameters (6V - 0.2-ms duration), and the time from 100% of the generated tension to 50% of the generated tension was calculated as ½ RT.

Force / velocity protocol

A force/velocity curve for the GS was determined after the test for maximum tetanic tension. The same setup as described above was used except that the load was adjusted to 12 different loads (~2 – 100% P_O) and change in length and speed of shortening was determined directly. Velocity of shortening was computed as a function of the distance shortened by the muscle over time (from 10-90% of peak tension) at each load. The least squares technique was used to find the best fit for a linearized version of the Hill equation (1938). Maximal shortening velocity (V_{MAX}) was determined by solving this equation for velocity when force equals zero.

Tissues Removal

After contractile properties were determined, the right non-stimulated extensor digitorum longus (EDL) muscle and it's tendon were dissected free and immediately placed in an oxygenated bath to be prepared for incubation. The muscles of the right hindlimb were analyzed for oxidative stress. Animals were euthanized with a lethal dose of sodium pentobarbital given IP. The SOL and GS were dissected free, immediately place in cold antioxidant buffer (100µm EDTA, 50mM Na_2HPO_4, 1mM BHT), blotted dry, weighed and frozen for biochemical analysis. The muscles of the left hindlimb were analyzed for protein concentration and water content.

Biochemical Analysis

Wet/dry weight

Approximately 25 mg of tissue was removed from the frozen SOL and GS samples. A precise frozen wet weight was measured, and then tissues were placed in a freeze-dry unit (Virtis Sentry Benchtop 3L). After reaching a steady dry weight, muscles were compared based on their frozen-wet weight to their dry weight.

Isolation of myofibrillar protein

Myofibrillar protein was isolated in SOL and GS muscles using a modification of the myofibril extraction technique described by Solaro et al. (1971); myofibrillar protein concentration was then determined using the biuret technique of Watters (1978). Precisely 100 mg of frozen muscle was homogenized in 2 ml of sucrose buffer and centrifuged for 10 min. at 1000 x g. The supernatant was discarded and the pellet suspended in a KCl buffer containing Triton X-100 to eliminate membrane ATPase components. The pellet was again homogenized and centrifuged at 1000 x g. This process was repeated in a KCl buffer yielding a myofibrillar protein pellet of sufficient purity for quantitative assessment of myofibrillar concentration.

Thiobarbituris acid-reactive substance (TBARS)

To determine the amount of ROS-mediated oxidative damage in the muscle, malondialdehyde (MDA), a by-product of lipid peroxidation, was measured. MDA levels were determined spectrophotometrically using TBARS method previously described by Uchiyama and Mihara (1978) with 1,1,3,3-tetramethoxypropane used as the standard.

Protein carbonyls

Oxidative damage to proteins is accompanied by increased levels of protein carbonyls (Yan et al., 1996; Oliveret al, 1987; Amici et al., 1989). Spectrophotometric detection and quantification of protein carbonyls is facilitated by the reaction of dinitrophenylhydrazine (DNPH) with protein carbonyls to form protein hydrazones. To quantify the amount of protein oxidation that occurred during HLU, total protein carbonyl derivatives were measured spectrophotometrically as described by Reznick and Packer (1994), with modifications reported by Yan et al. (Yan et al., 1996). Briefly, tissue protein was extracted in a protease-inhibitor (0.5μg/ml leupeptin, 0.7 μg/ml antipain, 0.5 μg/ml aprotinin, 40 μg/ml phenylmethylsulfonylflouride, one mM EDTA) and treated in 100mM potassium phosphate buffer, pH 7.4. Nucleic acids were removed from samples with a 1% streptomycin sulfate treatment. The samples were treated with one mM dinitrophenylhydrazine (DNPH). The protein was washed in ethyl actate-ethanol (1:1 vol/vol) and dissolved in 6 M guandidine hydrochloride, pH 2.3. Tissue protein carbonyl content was quantified by scanning the samples from 350 to 390nm in a spectrophotometer. The peak absorbance was used to calculate protein carbonyl content (extinction coefficient 22,000 l/mol/cm).

In Vitro Measurement of Tissue Antioxidant Capacity

To evaluate the ability of the supplement to increase the antioxidant capacity of the muscles, gastrocnemius homogenates from all four groups were subjected to three different ROS generating systems and then analyzed for lipid peroxidation using the TBARS assay. A section of the right gastrocnemius was homogenized at a concentration of 10:1 in either 50 mM potassium phosphate buffer (for the aqueous generating systems) or in ethanol (for the AMVN system) according to the method of Haramaki et al. (1995). Aliquots of the homogenates were incubated at a concentration of 10 mg protein/ml in the presences or absence of an ROS generating system. The following is a brief desscription of the three systems that were used.

Hydroxyl Radical System

Hydroxyl radicals were generated by an iron catalized system according to the method of Bernier et al. (1986). Briefly, 200 μl of 400 μm Ferrous Sulfate ($FeSO_4$) was added to 200 μl of homogenate and incubated at 37^0C for 15 mins. TBARS formation was then analyzed as previously described.

Hypoxanthine-Xanthine Oxidase System

Superoxide radicals were generated by a hypoxanthine-xanthine oxidase system according to the method of Fridovich (1970). Two hundred µl of one mM xanthine and 0.1 IU of xanthine oxidase were added to a 200 µl of homogenate and incubated at 37^0C for 120 mins. TBARS formation was then analyzed as previously described.

Peroxyl Radical System

Peroxyl radicals were generated in lipids in the gastrocnemius homogenate by thermal decomposition of 2,2 axobis 2-4 dimethylvaleronitrile (AMVN) according to method of Kagan et al. (1992). Two hundred µl of five mM AMVN was added to 200 µl of homogenate and incubated at 37^0C for 120 mins.

Following incubation of the gastrocnemius homogenates in each system, 200 mM butylated hydroxytoluene (BHT) and 1 mM deferioxamine in ice cold tricholoracetic acid (20% w/w) was added to stop the oxidative reaction. TBARS formation was then analyzed as previously described.

Statistical Analysis

All dependent measures (i.e. maximum tetanic tension, V_{MAX}, ½ RT, wet muscle weight, wet/dry muscle ratio, and biochemical parameters) were subjected to a one-way analysis of variance with a Scheffe test used post-hoc. Significance was established at $p<0.05$

CHAPTER 3
RESULTS

Overview of Experimental Findings

These experiments examined the individual and combined effects of antioxidant supplementation on disuse atrophy associated with hindlimb unweighting. The major findings of the study were that protein carbonyls were elevated as a result of hindlimb unweighting (figure 2) and supplementation with antioxidants did not attenuate the disuse atrophy associated with hindlimb unweighting (figure 5).

In an effort to determine the effectiveness of the antioxidant supplementation, a series of experiments were conducted that oxidatively challenged muscle from both the control diet and antioxidant supplemented animals. Our data indicate that the muscle from the antioxidant supplemented animals sustained significantly less oxidative damage when exposed to a range of reactive oxygen species (figure 10).

Although there is evidence of oxidative stress to muscle during hindlimb unweighting and that supplementation increased the muscle ability to buffer reactive oxygen species, antioxidant supplementation had no effect on the ability to attenuate atrophy associated with hindlimb unweighting (figure 5). Details of experimental findings are outlined in the following sections.

Antioxidant Supplementation

Animals tolerated the antioxidant supplementation well, no differences ($p<0.05$) in food intake existed between the control diet and antioxidant supplemented groups. No side effects of antioxidant supplementation were noted. Antioxidant intake is summarized in Table 3.

Table 3. Food and antioxidant intake

Group:	**Control**	**Antioxidant**	**Control Hindlimb Unweighted**	**Antioxidant Hindlimb Unweighted**
Pre Body Weight (g)	281.18	282.55	281.3	280.91
Avg. food intake (g/day)	17.19	16.67	16.98	16.87
Vitamin E (IU/day)	1.29	16.67	1.27	16.87
Selenium (mg/day)	0.0029	0.005	0.0029	0.0051
Vitamin C (mg/day)	0	16.67	0	16.87
β-carotene (mg/day)	0	3.75	0	3.79
N-acetyl cysteine (mg/day)	0	3.33	0	3.37
Catechin (mg/day)	0	1.67	0	1.69
Coenzyme Q_{10} (mg/day)	0	0.50	0	0.51
Vitamin E injection (IU/day)	0	8.48	0	8.43

Protein Carbonyls

Protein carbonyls were assayed as a measure of protein oxidation in the soleus muscle of all experimental groups. Soleus protein carbonyl concentrations were significantly ($p<0.05$) increased for both HLU groups as compared to weight bearing groups. Comparison of control diet groups to their respective antioxidant supplemented group indicate no difference ($p<0.05$) in carbonyl concentrations (figure 2).

Lipid Peroxidation

TBARS assay was used as a measure of lipid peroxidation in the gastrocnemius muscle of all experimental groups. Gastrocnemius TBARS measurement did not significantly differ ($p<0.05$) between experimental groups (figure 3).

Wet Muscle Weights

Muscle weights were taken as measurements of muscle atrophy. Soleus and gastrocnemius wet weights of the hindlimb unweighted groups decreased significantly ($p<0.05$) compared to the respective weight bearing groups (figure 5). Further, no significant difference ($p<0.05$) was demonstrated between control diet and antioxidant supplemented groups for either the soleus or gastrocnemius (figure 5).

Gastrocnemius Contractile Function

In Situ gastrocnemius contractile function was assessed at the end of the experimental period. Absolute tetanic and twitch forces were significantly decreased ($p<0.05$) in the hindlimb unweighted groups as compared to the weight bearing groups while the antioxidant supplemented groups were not significantly different ($p<0.05$) from the control diet groups respectively (figure 6). When comparing specific tension (g tension / g muscle weight), no significant difference ($p<0.05$) existed between any groups for both the tetanic and twitch measurements (figure 7). No significant difference ($p<0.05$) was demonstrated for either ½ relaxation time (figure 8) or maximum velocity of contration (V_{MAX})(figure 9).

Muscle Protein Concentration and Water Content

Muscle protein concentrations (mg protein/g muscle) and water content (expressed as a percentage of total muscle) did not significantly differ among experimental groups (table 4).

Table 4. Muscle protein concentration and water content

Muscle:	Group:	Water Content %	Total Protein mg/g	Myofibrillar Protein mg/g	Soluble Protein mg/g
SOL	Control	78.7 ± 0.3	204.0 ± 6.9	117.1 ± 6.3	86.8 ± 3.0
	AO	76.9 ± 0.4	206.4 ± 6.2	118.7 ± 6.0	87.7 ± 1.3
	HLU-C	77.3 ± 0.6	202.1 ± 6.1	114.4 ± 5.1	87.7 ± 3.2
	HLU-AO	78.3 ± 0.4	209.9 ± 4.6	122.4 ± 4.1	87.5 ± 2.8
GS	Control	78.6 ± 0.7	194.3 ± 4.1	116.5 ± 5.7	77.8 ± 3.1
	AO	79.5 ± 0.7	197.4 ± 3.5	115.0 ± 4.7	82.4 ± 4.5
	HLU-C	77.8 ± 0.4	197.4 ± 4.9	112.5 ± 5.6	84.9 ± 3.6
	HLU-AO	78.4 ± 0.8	199.1 ± 3.4	121.6 ± 3.7	77.5 ± 4.6

In Vitro Oxidative Challenges

In Vitro oxidative challenges were employed to evaluate the ability of the supplement to increase the antioxidant capacity of the muscles. The gastrocnemius from the supplemented groups was better protected ($p<0.05$) against lipid peroxidation in the two aqueous radical generating systems compared to the gastrocnemius from control diet groups (figure 10). Similarly, compared to control, gastrocnemius from antioxidant supplemented animals experienced less ($p<0.05$) lipid peroxidation when exposed to the lipid phase (AMVN) oxidative challenge (figure 10).

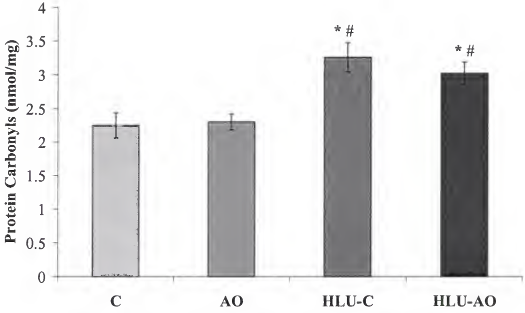

Figure 2. Soleus Concentration of Protein Carbonyls; values are means ± SEM; * indicates greater than control diet weight bearing ($p<0.05$); # indicates greater than antioxidant supplementation weight bearing ($p<0.05$)

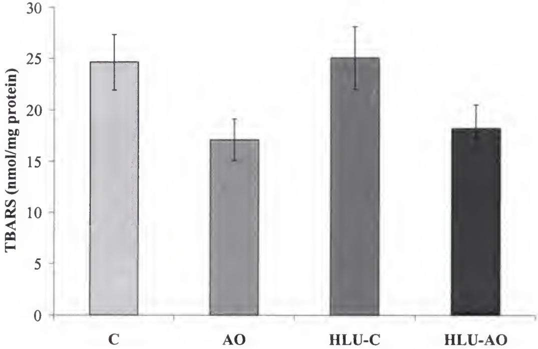

Figure 3. Gastrocnemius concentration of lipid peroxidation (TBARS assay); Values are means ± SEM; No significant difference ($p<0.05$) between groups.

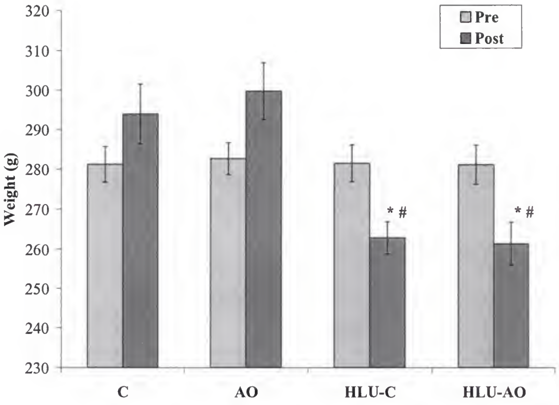

Figure 4. Body Weight; values are means + SEM; * indicates different that control diet weight bearing ($p<0.05$); # indicates different than antioxidant supplementation weight bearing ($p<0.05$).

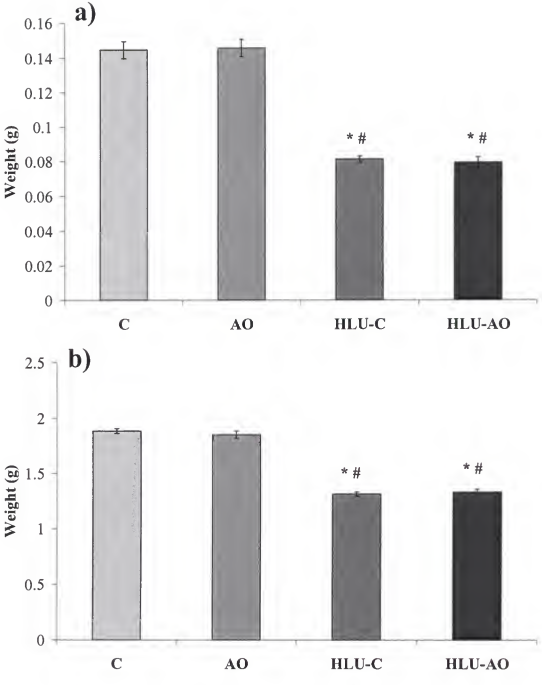

Figure 5. Wet Muscle Weights; (a) Soleus; values are means ± SEM; * indicates different than control diet weight bearing ($p<0.05$); # indicates different than antioxidant supplementation weight bearing ($p<0.05$); (b) Gastrocnemius; values are means ± SEM; * indicates different than control diet weight bearing ($p<0.05$); # indicates different than antioxidant supplementation weight bearing ($p<0.05$).

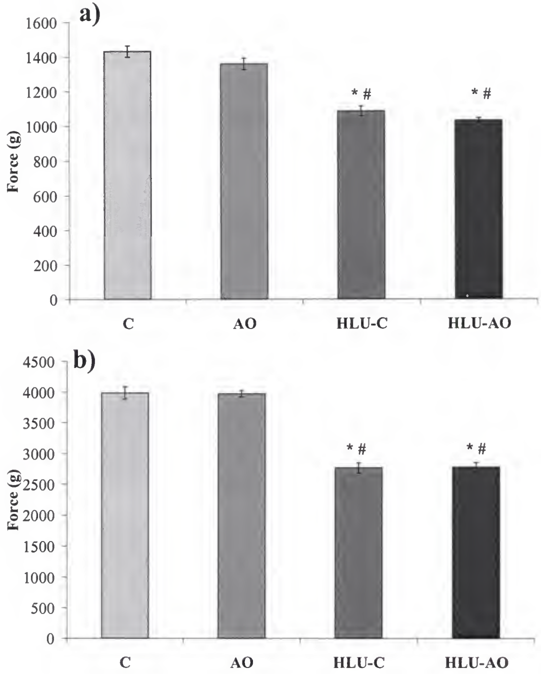

Figure 6. *In Situ* Gastrocnemius Absolute Force Production; (a) Twitch Force; values are means ± SEM; * indicates different than control diet weight bearing ($p<0.05$); # indicates different than antioxidant supplementation weight bearing ($p<0.05$); (b) Maximal Tetanic Force; values are means ± SEM; * indicates different than control diet weight bearing ($p<0.05$); # indicates different than antioxidant supplementation weight bearing ($p<0.05$).

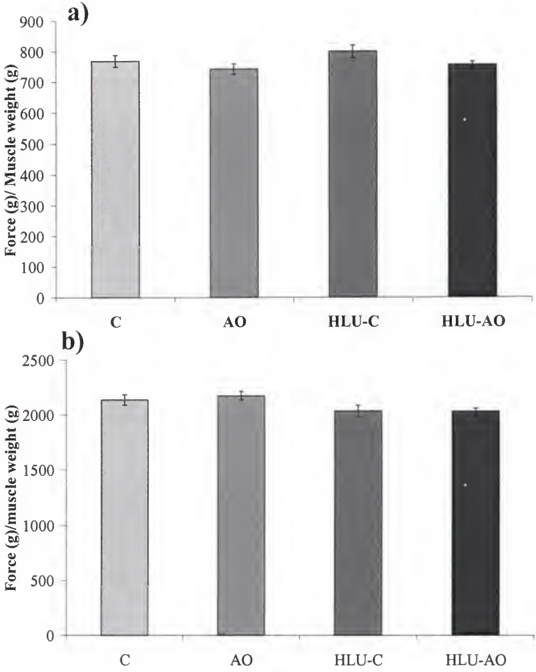

Figure 7. *In Situ* Gastrocnemius Specific Tension; (a) Twitch Force; Values are means ± SEM; No significant difference between groups ($p<0.05$); (b) Maximal Tetanic Force; Values are means ± SEM; No significant difference between groups ($p<0.05$)

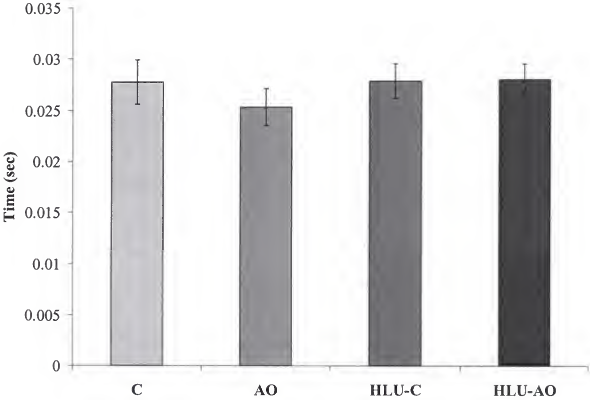

Figure 8. ½ Relaxation Time; values are means + SEM; No significant difference between groups ($p<0.05$).

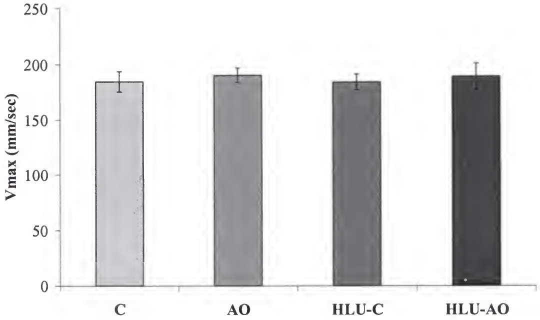

Figure 9. Maximum Velocity of Contraction; values are means ± SEM; No significant difference between groups ($p<0.05$).

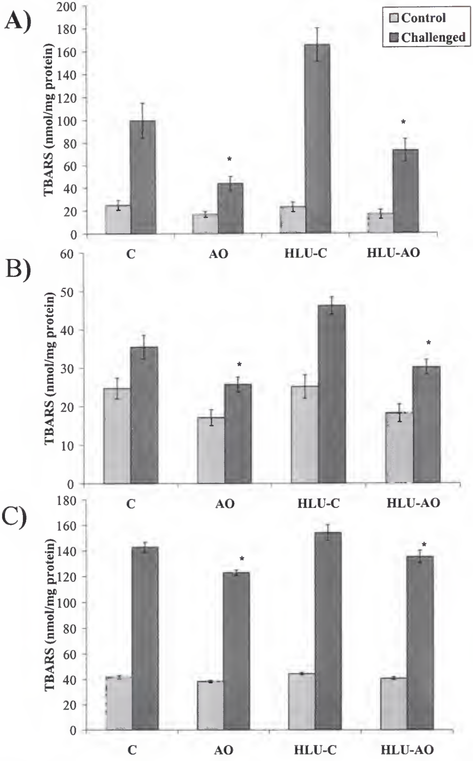

Figure 10. Lipid peroxidation (TBARS assay) in gastrocnemius homogenate subject to *in vitro* oxidative challenges; Values are means ± SEM; * indicates significant decrease ($p<0.05$) compared to coresponding control diet group; (a) Iron challenge; (b) Xanthine oxidase challenge; (c) AMVN challenge

CHAPTER 4
DISCUSSION

Overview of Experimental Findings

This is the first study to examine the effects of an antioxidant supplementation on disuse atrophy associated with hindlimb unweighting. The major findings of this study were that protein oxidation increased significantly ($p<0.05$) with HLU. Muscle weight, body weight and absolute force all decreased ($p<0.05$) which is consistent with other HLU studies. Although the antioxidant supplementation did not attenuate the disuse atrophy associated with HLU, *in vitro* experiments demonstrated antioxidant protection of the gastrocnemius against lipid peroxidation induced by three different radical generating systems. Together these data indicate that the antioxidant supplementation was not an effective countermeasure to the disuse atrophy associated with hindlimb unweighting.

Antioxidant Supplementation and Skeletal Antioxidant Capacity

The antioxidant supplementation used in these experiments was modeled after supplementation used by others who demonstrated protection of a variety of tissues against oxidative injury (Chen & Tappel, 1994; Knudsen et al., 1996; North & Tappel, 1997). The supplementation protocol used in this study resulted in signficantly ($p<0.05$) increased antioxidant protection. This was supported by the finding that, after exposure to three ROS generating systems, lower levels of TBARS were detected in skeletal muscle homogenates of supplemented animals compared to control diet groups. Kondo and Itokawa (1994) and Appell et al.(1997) demonstrated attenuation of the disuse atrophy associated with immobilization by supplementation with vitamin E. However, no attempts were made to evaluate if the treatment elevated vitamin E levels or antioxidant protection in the muscle. The findings of this study, compared to those of Kondo and Appell, suggest that the mechanism governing muscle wasting in immobilization and unweighting may be fundamentally different.

Protein Oxidation and Lipid Peroxidation

To our knowledge this is the first study to evaluate the level of protein oxidation and lipid peroxidation post HLU. In the soleus, protein carbonyls, a measure of protein oxidation, were elevated in the HLU group and antioxidant supplementation did not attenuate this increase. The increase in protein carbonyls with the increased antioxidant capacity of the muscle would indicate that disuse muscle atrophy is not related to the antioxidant capacity of the muscle.

TBARS assays, a measure of lipid peroxidation, in the gastrocnemius was not different between groups. Although Kondo (1994) demonstrated an increased TBARS, a marker of lipid peroxidation, with immoblization, the data in this study do not indicate an increased in lipid peroxidation with HLU. Jaspers et al. (1988 & 1989) had demonstrated different responses of skeletal muscle to immobilization and HLU, which indicate that the mechanism for degradation and/or oxidation may be different. Whether the conditions that lead to protein oxidation also increase lipid peroxidation have yet to be determined. A number of studies on blood, liver, heart, testes, brain and skeletal muscle have also demonstrated different results between TBARS and protein carbonyls (Alessio, 1999; Youngja, 1991; Lucesoli, 1999; Lyras, 1997; Oteiza, 1997; Lass, 1998;

Zamora,1997). These studies and the current data would indicate that different mechanisms may be involved in protein and lipid oxidation, and further research would be warranted to investigate the possible mechanisms of protein and lipid oxidation with HLU.

Muscle Weight and Body Weight

Muscle weights decreased 44.5% and 29.2% for SOL and GS respectively. This finding is consistent with the theory that HLU causes atrophy, with the greatest change observed in antigravity muscles such as the soleus (Fitts et al., 2000; Caiozzo et al., 1994; Widrick et al., 1999). Antioxidant supplementation did not effect the degree of atrophy for SOL (45.8%) and GS (28.4%). This finding is not consistent with that of Kondo and Itokawa (1994) and Appell et al. (1997), where they demonstrated attenuation of atrophy due to immobilization with the administration of vitamin E.

The body weight gain of weight-bearing antioxidant supplemented (17.1 ± 5.2g) and control diet (12.7 ± 4.0g) groups were similar. Furthermore, the body weight loss of HLU antioxidant supplemented (-19.7 ± 4.4g) and HLU control diet (-18.6 ± 2.9g) groups were consistent and were in the range of other studies (Reiser et al., 1987; Naito et al., 2000). Average daily food comsumption was consistant between all groups (c-17.2 ± 0.5; AO-16.7 ± 0.6; HLU-C 17.0 ± 0.4; HLU-AO 16.9 ± 0.5). These data indicate that the muscle mass and body weight loss observed in the HLU groups was mainly related to unweighting conditions and that the antioxidant supplementation did not effect the amount of atrophy due to HLU.

Contractile Properties

The mixed fiber type GS was used to determine variations of *in situ* contractile properties. The absolute twitch and tetanic force production decreased for the HLU control diet group (24.4% & 30.8%). These data are consistent with decreases in muscle mass with other hindlimb unweighting studies (Fitts et al., 2000; Diffee et al., 1991; Caiozzo et al., 1994; Templeton et al., 1984). The antioxidant supplementation did not attenuate the decreases in twitch (24.2%) and tetanic (30.1%) forces. Specific tension was unaltered as a result of HLU, which is consistent with the findings of Fitts et al. (2000), Diffee et al. (1991) and Fell et al. (1985).

Although it is well accepted that V_{MAX} will increase for the SOL as a result of HLU, alteration in half relaxation time and V_{MAX} of other muscles have not been characterized (Fitts et al., 2000; Diffee et al., 1991; Caiozzo et al., 1994). Our data demonstrate no change in ½ RT or V_{MAX} for any of the experimental groups. The mixed GS may not experience the same extent of slow to fast shift in muscle type as demonstrated in the slow SOL (Fitts et al., 2000; Diffe et al., 1991; Caiozzo et al., 1994) and may explain why no change in V_{MAX} for the GS was demonstrated. No change in the ½ RT compares with the findings of others data (Fitts et al., 2000; Diffee et al., 1991; Caiozzo et al., 1986; Fell et al., 1985).

Protein Concentration and Water Content

Muscle mass loss, and the decreased force production that accompanies it, may be due to a decrease in muscle protein and / or water. Our data indicate that there is no change in protein concentration or water content. This agrees with the findings of Fell et al. (1985), Jaspers and Tischler (1986), and Naito et al. (2000) and suggest that all components of muscle are lost in equal proportions.

Why No effect from Antioxidant Supplementation

The mechanisms of atrophy may be fundamentally different between immobilization and HLU. Immobilization does not permit for any movement, but does allow the muscle to generate an isometric force, where as, HLU permits a free range of motion, but does not offer any resistance against which the muscle can generate any force. Jaspers et al. (1988) demonstrated that immbolization in the stretched postion of unweighted soleus attenuated atrophy. This mechanism is thought to be due to increased internal tension in the muscle from the stretching. Therefore, the lack of force generation with HLU may affect in a different mechanism of atrophy versus immobilization. It is important to note that other studies that examined muscle wasting have used the immobilization model.

It is unlikely that the antioxidant supplementation may not have protected against oxidative stress. As stated earlier, after exposure to three ROS generating systems (iron to generate the hydroxyl radical, xanthine oxidase to generate the superoxide radical & AMVN to generate the peroxyl radical), lower levels of TBARS were detected in the skeletal muscle homogenates of the antioxidant supplemented animals compared to control diet groups. These data indicate that the antioxidant supplementation resulted in a significant increase in antioxindat protection in the muscle.

Oxidation of proteins may not be causing the protein degradation. The elevated protein carbonyls may have been due to activation of other degradation pathways not involving free radicals. Berlett and Stadtmann (1997) state that the accumulation of oxidized protein reflects not only the rate of protein oxidation but also the rate of oxidized protein degradation, which is also dependent on many variables, including concentrations of proteases that preferentially degrade oxidized proteins and numerous factors (metal ions, inhibitors, activators, and regulatory proteins) that affect their proteolytic activity. For example, oxidized forms of some proteins (e.g. cross-linked proteins) and proteins modified by glycation or lipid peroxidation products are not only resistant to proteolysis but, in fact, can inhibit the ability of proteases to degrade the oxidized forms of other proteins (Berlett and Stadtman, 1997).

Alterations in calcium homeostasis may have an effect on protein degradation. Ingalls et al. (1999) demonstrated that unloading of the soleus increased the resting calcium concentration 36% above control. One mechanism that may allow the influx of calcium is the L-type calcium channels which open during passive shortening (Wang et al., 2000). Increased calcium can activate calpains which seem to degrade structural proteins (e.g. titin) during muscle disuse (Taillandier et al. , 1996). Opening the way for the ubiquitin proteasome pathway which is an important system responsible for the breakdown of long-lived skeletal muscle proteins during disuse including the major contractile proteins (e.g. myosin and actin) (Furuno et al., 1990; Taillandier et al. , 1996). Measurments of calcium levels resulting from HLU would offer further information toward the hypothesis that alterations in calcium homeostasis may have an effect on protein degradation.

Summary and Conclusions

This is the first study to examine the effects of antioxidant supplementation on disuse atrophy associated with hindlimb unweighting. An increase in protein oxidation, indicated by an increased level of protein carbonyls, was demonstrated with HLU. Changes in muscle weight, body weight and contractile properties were consistent with exposure to unweighting. Antioxidant supplementation did not attenuate the disuse atrophy resulting from HLU. The use of oxidative challenges to the muscle illustrated that there was an increased antioxidant protection. These data indicate that the antioxidant supplementation was not an effective countermeasure to the disuse associated with HLU.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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